Developmental Cell, Vol. 1, 1–20, October, 2001, Copyright ©2001 by Cell Press

Signaling Pathways in Mammary Gland Development

Review

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Unlike most other organs, development of the mammary gland occurs predominantly after birth, under the control of steroid and peptide hormones. Once the gland is established, cycles of proliferation, functional differentiation, and death of alveolar epithelium occur repeatedly with each pregnancy. Although it is unique in this respect, the signaling pathways utilized by the gland are shared with other cell types, and have been tailored to meet the needs of this secretory tissue. Here we discuss the signaling pathways that have been adopted by the mammary gland for its own purposes, and the functions they perform.

A Historical Perspective

During the past 100 years, extensive efforts have been made to investigate the regulation of mammopoiesis and to understand the endocrine signals and pathways that control mammary epithelial cell proliferation and differentiation. Advances in the first three decades of the 20th century were based on the development of techniques for multiple endocrinectomies and the isolation of bioactive hormones. Evidence for ovarian control of mammary development first emerged 100 years ago, when Halban (1900) and Knauer (1900) demonstrated that oophorectomy (ovary removal) caused mammary regression and that transplanted ovaries prevented this atrophy. The responsible bioactive compounds turned out to be progesterone and estrogen (Allen et al., 1924). When hypophysectomy (removal of the pituitary gland) was a routine experimental procedure in the 1920s, it became clear that factors other than ovarian hormones were required for mammopoiesis. The era of peptide hormones in mammary development was born in 1928 when Stricker and Grueter in Strasbourg induced milk secretion artificially in castrated virgin rabbits with pituitary extracts from lactating animals (Stricker and Grueter, 1928). Five years later, Riddle and his colleagues from the Carnegie Institution of New York (now the Cold Spring Harbor Laboratory) purified the respective pituitary hormone (Riddle et al., 1933) and named it prolactin (Prl). As early as 1906 it became evident that the placenta also secretes mammotrophic substances (Lane-Claypon and Starling, 1906), which include placental lactogens, estrogens, progesterone, and gonadotrophins. The introduction of mammary organ cultures that could be maintained in vitro in chemically defined media supplemented with a cocktail of hormones revealed that synergistic action of insulin, hydrocortisone, and prolactin controls the differentiation of secretory mammary epithelium (Topper and Freeman, 1980).

The cloning of steroid and peptide hormone receptors in the 1980s, the identification of downstream signaling components in the 1990s, and the isolation of target genes provided a basis for the mechanistic understanding of signal transduction pathways. Further, the use of experimental mouse genetics has been instrumental not only in confirming the roles of the candidate genes but also in the discovery of new signaling pathways in the mammary gland. Among the genes identified to control mammary development were some of the "usual suspects" but also genes that had not been linked previously to mammary gland development. Finally, the combination of experimental mouse genetics with experimental tissue recombination has provided evidence for cell-autonomous signaling pathways and signaling cascades that depend on crosstalk between epithelial and stromal cells in the developing organ (e.g., Robinson et al., 2000).

Development and Structure

Development of the mammary gland occurs in defined stages that are connected to sexual development and reproduction. These are embryonic, prepubertal, pubertal, pregnancy, lactation, and involution. Two cellular compartments contribute to the gland, the epithelium and the surrounding stroma, which are derived embryologically from ectoderm and mesoderm, respectively. The nipple is derived from mammary mesoderm. The epithelium consists of a branched ductal system that develops mainly during puberty (Figure 1d) and a lobuloalveolar compartment that develops during pregnancy (Figure 1e). The ducts branch into decreasingly smaller ductules, which terminate in lobules. Lobules are composed of alveoli, which in turn consist of secretory epithelial cells that undergo functional differentiation with parturition. The ducts are surrounded by a continuous layer of contractile myoepithelial cells and the alveoli have a more open network of myoepithelium. These cells contract in response to oxytocin stimulation, which results in milk release.

Epithelial-Mesenchymal Signaling during Embryonic Development

The initial stages of mammary development are independent of systemic cues and instead depend on reciprocal signaling between the epithelium and the mesenchyme. In the mouse embryo, five pairs of ectodermal placodes appear between embryonic days 10 and 11. They form as distinct spots in two lines running ventrally just inside the limbs from the neck to the genital area. The placodes form buds, which slowly increase in size up to embryonic day 15 (Figure 1a). During this period, the epithelial bud is surrounded by a halo of layered mesenchymal cells, the primary mammary mesenchyme. Cell proliferation intensifies at the tip of the bud

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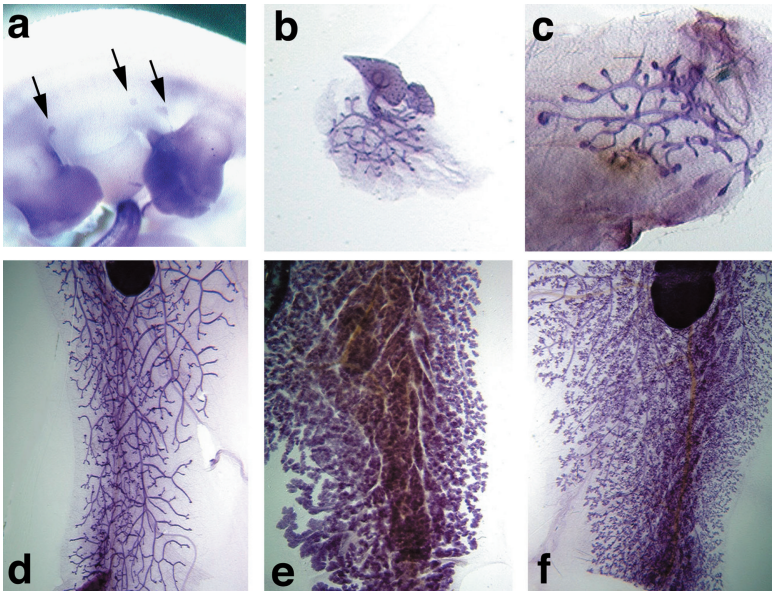

Figure 1. Fetal and Adult Stages of Mammary Gland Development

Development of the mammary gland begins in the fetus. The anlagen (arrows) are visualized by whole-mount in situ hybridization with PTHrP (a). A small ductal tree is present in newborn animal (b); the arrow points to the nipple region. The size and branching pattern of the ductal system increases only moderately until the onset of puberty at 3 weeks (c); the arrow points to a terminal end bud (TEB). Accelerated growth and branching induced by puberty hormones leads to the formation of an extended ductal system that fills the entire fat pad in a mature virgin mouse (d). Alveolar development occurs during pregnancy (e). The alveolar epithelial compartment is eliminated during involution (f).

and leads to the formation of a primary sprout, which grows out of the surrounding mesenchyme toward the prospective mammary fat pad. This fat pad forms the stroma of the adult mammary gland. Continued proliferation and branching leads to the formation of a small ductal tree at birth (Figure 1b). Mesenchyme from the prospective mammary region is able to induce mammary epithelial differentiation when combined with dorsal epidermis (Cunha et al., 1995), demonstrating that a mesenchymal signal first determines epithelial development. As in teeth and hair, reciprocal interactions between the two tissues are critical for further development of the gland. Once the early bud is initiated, it induces the formation of a characteristic primary mammary mesenchyme in adjacent mesenchymal cells (Figure 2). The "primary mammary mesenchymal cells" are morphologically distinct from the more distant dermal mesenchyme and express receptors for estrogen and androgen, the transcription factors Lef-1, Msx1, and Msx2, the growth factors BMP4 and FGF7, and the extracellular matrix molecules tenascin C and syndecan-1 (for review see Robinson et al., 1999). Tissue recombination experiments have demonstrated that the capacity of the mammary epithelium to induce these mesenchymal markers is transient and is lost at later stages (Heuberger et al., 1982).

Lef-1, Msx1, and Msx2 expression is not unique to the early mammary anlage and is also found in teeth, hair, and whiskers. Therefore, it does not come as a surprise that the inactivation of the genes encoding these transcription factors leads to arrest of the development of all these ectodermal appendages, including the mammary gland, at the bud stage (Figure 3; Table 1). In the mammary gland, Lef-1 RNA is first found at sites where the placodes are formed (van Genderen et al., 1994), followed by a shift to the mesenchyme (Satokata et al., 2000). The genes encoding Msx1 and Msx2 initially are coexpressed in the developing placodes (Phippard et al., 1996). At a slightly later stage, *Msx1* gene expression is downregulated, whereas *Msx2* gene expression occurs also in the mesenchyme (Satokata et al., 2000). Mammary gland development is not affected by the absence of Msx1 alone. However, mice in which both Msx1 and Msx2 have been inactivated lack mammary buds (Satokata et al., 2000). By contrast, Msx1 alone is required for tooth development beyond the late bud stage. This suggests cell- and organ-specific differences in the expression levels and patterns of these transcription factors and/or differences in the signaling pathways elicited in the recipient mesenchyme. Tissue recombination experiments in tooth buds and whiskers have shown that the requirement for these transcription factors is temporally restricted and their action is noncell autonomous (Kratochwil et al., 1996). Transient expression is required in one tissue to induce changes in the other tissue to promote development of the organ. The development of Msx1-deficient tooth buds is arrested at the late bud stage but can be rescued by treatment with BMP4 (Bei et al., 2000), suggesting that this is the epithelial signal that causes differentiation of the dental papilla mesenchyme. No specific downstream signals activated by Msx-1 and 2 have been identified in the mammary gland. It will be interesting to see whether they are identical in teeth, hair, and mammary glands or whether different responses ensue in a tissue-specific context reflecting the differences in morphology and differentiation of the organs.

Mesenchymal-epithelial signaling through PTHrP (parathyroid hormone related peptice) and its receptor provides important cues for the elongation of the primary sprout (Figure 2). PTHrP is expressed in mammary epithelium, and the signal is received by the surrounding mesenchymal cells, which express the receptor. Interruption of PTHrP signaling causes developmental arrest of the mammary gland primordium before elongation of the bud into a primary sprout begins. Identical phenotypes were observed in mice lacking the ligand PTHrP or the PTH/PTHrP receptor PPR1. The arrest occurs 4 days after PTHrP expression begins in the epithelial mammary bud (Wysolmerski et al., 1998). In the absence

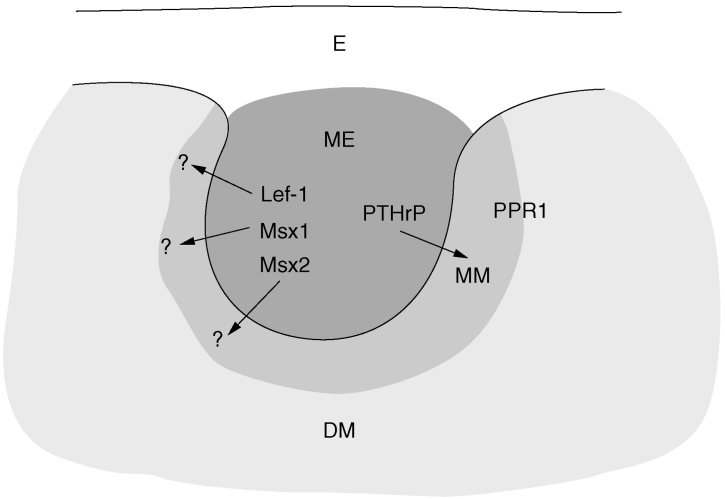

Figure 2. Stromal and Epithelial Signals Operative in the Fetal Anlage

Genetic and tissue transplant experiments have implicated the genes listed. The primary mammary mesenchyme (MM), which surrounds the mammary epithelial bud (ME), is distinct from the dermal mesenchyme (DM) (light gray). E, epidermis.

of the signal, markers for the primary mammary mesenchyme are not expressed. Most noticeable is an absence of the androgen receptor and tenascin C expression, whose expression can be restored by expression of a *PTHrP* transgene in the knockout mice (Dunbar et al., 1999). Similarly, ectopic expression of PTHrP in the epidermis causes the adjacent dermal cells to assume characteristics of primary mammary mesenchyme and differentiate as nipple cells (Foley et al., 2001). Thus, PTHrP is the first signaling molecule known to be produced by the embryonic mammary epithelial cells that influence cell fate decisions in the surrounding mesenchyme. These in turn evoke a proliferation and differentiation response in the epithelium. Lack of PTHrP signaling also affects bone development by regulating chondrocyte proliferation and differentiation and mice missing PTHrP or the PPR1 receptor show accelerated differentiation of chondrocytes (Chung et al., 1998). PTHrP also functions in the adult epidermis, and knockout mice display premature differentiation of keratinocytes, hypoplastic sebacious glands, and a fibrotic dermis (Foley et al., 1998).

Steroid Hormones Control Ductal and Alveolar Development

Following the embryonic and prepubertal stages, further development of the mammary gland becomes hormone-dependent and continues at the onset of puberty. The systemic steroid hormones that regulate this process were initially revealed by hormone depletion, through endocrine ablations and defined hormone reconstitution studies (for review see Imagawa et al., 1994). Gene-targeting approaches have identified specific and overlapping roles of the progesterone and estrogen receptors, plus other coactivators and transcription factors that mediate signaling processes in the developing mammary gland (Figure 3; Table 1). The primary mechanism of steroid hormone function is through binding to specific nuclear receptors, which activate defined genes in a ligand-dependent manner. ER and PR are found at high levels in ovaries, uterus, mammary, and pituitary glands. ERα null mice are infertile, and ductal development during puberty is severely curtailed. Impaired ductal development is not only the result of a lack of ER in the mammary epithelium and stroma, but also due to the failure of estrogen signaling through the hypothalamic/pituitary axis (Bocchinfuso et al., 2000). ERα null mice have reduced levels of prolactin, which results in a nonfunctional corpus luteum and insufficient progesterone synthesis to maintain pregnancies and execute mammary ductal development. Wild-type pituitary isografts were able to partially rescue the defects observed in ERα null mice, further emphasizing that mammary development is controlled to a large extent by systemic cues.

The physiological effects of progesterone are mediated by the interaction of two receptors (PR-A and PR-B) that are encoded by a single gene containing two distinct promoters. The PR-B isoform is identical to PR-A but contains additional 165 amino-terminal amino acids as the result of a new translational start site. Mice lacking both PR isoforms display pleiotropic reproductive abnormalities, including an inability to ovulate and severely limited mammary gland development (Lydon et al., 1995). Since ovarian dysfunction and a lack of signaling through the hypothalamic/pituitary axis significantly affect mammary development, mammary epithelial transplants were used to establish the direct function of PR in epithelial cells (Brisken et al., 1998). In the absence of the PR, alveolar development during pregnancy is completely absent. However, it is not known whether the PR and PrlR pathways act independently or are interdigitated. Since the PR-A and PR-B isoforms posses different transcriptional activities in vitro, unique in vivo functions were predicted. Specific mutation of the initiating ATG used in the PR-A form using Cre/loxP-based site-specific recombination resulted in mice that expressed the PR-B isoform only (Mulac-Jericevic et al., 2000). Like the PR null mice, PR-A null mice were infertile, partly due to defective uterine implantation. In these mice, ductal branching and alveolar budding was observed upon stimulation with estrogen and progesterone. Based on this assay, it appears that the PR-B isoform is sufficient for ductal outgrowth, branching, and the formation of alveolar lobulules. However, a bona fide proof of the role of either isoform will require mammary gland transplants or a cell-specific deletion of the individual isoforms. Although the pathways activated by progesterone are poorly understood, it is now clear that

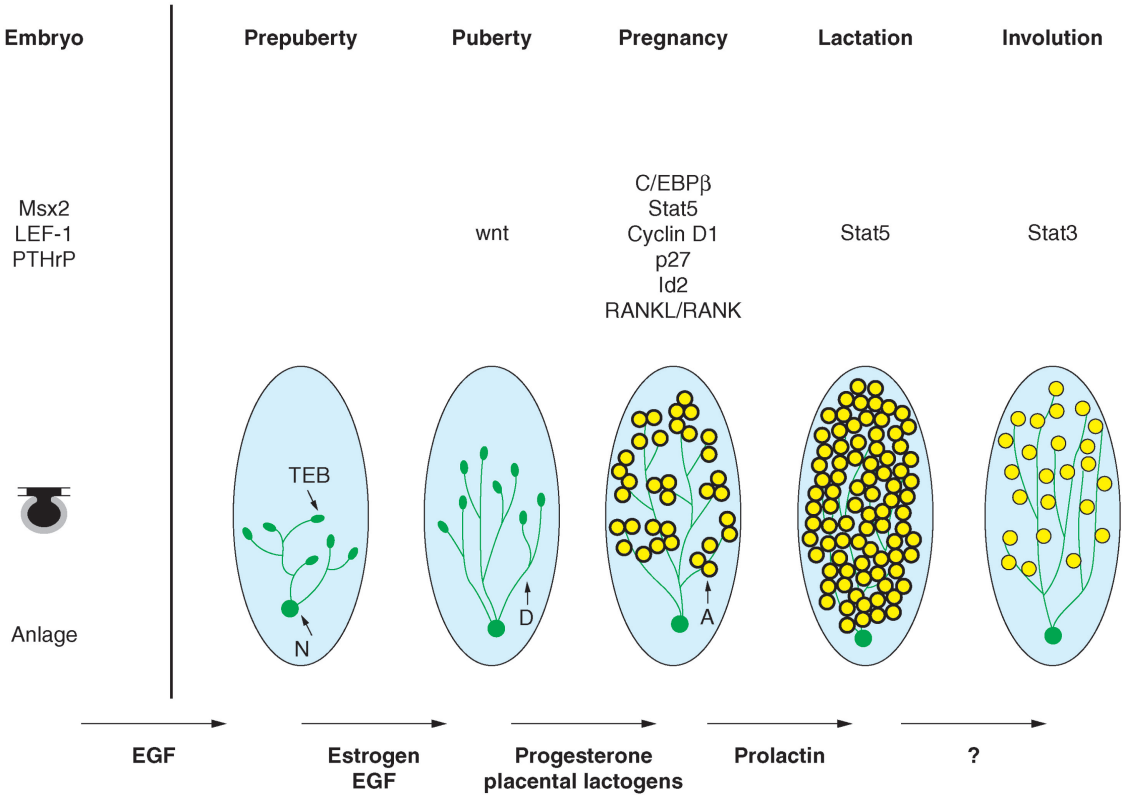

Figure 3. Hormones and Genes that Control Development of the Mammary Gland

The schematic drawing represents different developmental stages. The anlage is composed of epithelium (dark knob) and stroma (gray surrounding). The oval shown in the postnatal stages depicts the mammary fat pad (stroma). The solid green circle represents the nipple (N) from which the ducts originate. The ends of growing ducts form terminal end buds (TEB) during puberty. Mammary ducts (D) are shown as solid lines and the lobuloalveolar structures are presented as yellow circles (A). The hormones that control distinct developmental windows are shown below the arrows. The genes that have been implicated genetically in developmental processes are listed above the respective stages. Epidermal growth factor (EGF) signals through the stroma and controls early ductal outgrowth. Together with estrogen, it also controls ductal elongation and branching during puberty. Progesterone, placental lactogens, prolactin, and the osteoclast differentiation factor signal alveolar proliferation and differentiation during pregnancy and possibly lactation. The signals inducing tissue remodeling during involution have not been defined. The genes that control distinct stages of mammary development are shown. Ductal elongation and branching during puberty is controlled by inhibinβB, CSF-1, the progesterone receptor, and wnt. Proliferation and differentiation of mammary alveolar cells is controlled by the prolactin receptor, Stat5, RANKL, cyclinD1, p27, Id2, and C/EBPβ. Mammary function during lactation is controlled by prolactin through Stat5, and tissue remodeling and cell death during involution by Stat3, bax, and bcl-x.

it encompasses the control of cell proliferation through activating the wnt pathway in a paracrine fashion (Brisken et al., 2000). Estrogen and progesterone are primarily known for their role in the development and function of the female reproductive system. Since the need for a functional mammary gland was dependent on a successful pregnancy, it appears more than prudent for the evolutionary process to use the same set of hormones for both developmental processes.

Nuclear receptors require coactivators to mediate transcriptional activation of their target genes. The family of steroid receptor coactivators (SRC) consists of three members, SRC-1, GRIP1 (SRC-2), and p/CIP (SRC-3), which affect transcriptional activation through several mechanisms. These include direct interactions with ligand bound nuclear receptors and generic transcription factors, interactions with common transcriptional coactivators (CBP, p300), and participation in chromatin remodeling through their intrinsic histone acetyltransferase activity. *SRC-3* is highly amplified in 10% of primary breast cancers and its mRNA is overexpressed in the majority of primary breast cancers (Bautista et al., 1998), suggesting a role for SRCs in mammary development and breast cancer. Although in vitro assays seemed to indicate redundancy among SRCs, there is biological specificity in vivo. Outgrowth of mammary ducts during puberty was retarded in SRC-3 null mice (Xu et al., 2000). However, this was not the result of an intrinsic defect in the mammary epithelium but instead was a secondary effect. SRC-3 null mice have decreased levels of systemic estrogen, which result in delayed sexual maturation and thus may account for the majority of the ductal outgrowth defect. They also show decreased ovulation, lower pregnancy frequency, and small litter sizes, suggesting a general role of SRC-3 in female reproduction. Administration of pharmacological doses of estrogen and progesterone can induce ductal side branching in control mice, but SRC-3 null epithelium did not undergo branching to the same degree, suggesting some affects intrinsic to the mammary epithelium as well (Xu et al.,

Table 1. Genetic Pathways Controlling Mammary Development and Other Organs

Signaling Pathways	Mammary Defects	Reproductive System
Steroid Hormones		
Estrogen receptor α	Absence of ductal outgrowth	Infertile
Progesterone receptor	Impaired alveolar development	Infertile
SRC-1, SRC-3	Reduced ductal branching	Fertile
Peptide Hormone Pathways		
Prolactin	Reduced ductal branching	Infertile
Prolactin receptor	Reduced ductal branching, impaired alveolar development	Nonfunctional corpus luteum
Stat3	Delayed involution	ND
Stat5a	Impaired differentiation	None
Stat5b	None	Nonfunctional corpus luteum
Stat5a/b	ND	Nonfunctional corpus luteum
Bcl-x	ND	Loss of Primordial germ cells
RANKL or RANK	Impaired alveolar development	ND
PTHrP or PPR1	Lack of embryonic mesenchyme and prim. sprout growth	ND
Cell Cycle		
Cyclin D1	Impaired alveolar development	ND
p27	Impaired alveolar development	Infertile, nonfunctional corpus luteum
p27 and cyclin D1	Normal development	Same as p27
Id2	Impaired alveolar development	ND
Transcription Factors		
C/EBPβ	Impaired ductal and alveolar development	Lack of corpus luteum
LEF-1	Arrest at embryonic bud stage	ND
Msx1 and Msx2	Arrest at embryonic bud stage	ND

Signaling molecules that control proliferation, survival, differentiation, and death of mammary epithelial cells as determined through experimental genetics of the mouse. The genes that have been deleted using conventional and cell-specific recombination approaches are shown in the left column. The effects on mammary glands and the reproductive system are listed in the right columns. ND, not described.

2000). For a better understanding of the role of SRC-3, it will be necessary to investigate ductal and alveolar development during pregnancy in transplanted tissue. Deletion of SRC-1 resulted in an even milder phenotype (Xu et al., 1998). Females were fertile but showed a partial resistance to estrogen and progesterone. As in SRC-3 null mice, mammary development was slightly reduced in the absence of SRC-1, and most of this effect can be attributed to insufficient steroid signaling through the ovary. Thus, although individual steroid receptor coactivators can significantly modulate the transcriptional activity of steroid hormone receptors in vitro, their individual roles appear to be more restricted in vivo. The presence of several coactivators and some degree of functional redundancy may obscure the full potential of each molecule in vivo.

The importance of hormone receptors in cell proliferation is further emphasized by results obtained in an apparently unrelated knockout model. Inactivation of the transcription factor C/EBPβ results in reduced ductal growth and morphogenesis and abrogation of alveloar differentiation in an epithelial cell autonomous way (Robinson et al., 1998; Seagroves et al., 1998). In C/EBPβ null mammary glands, a much higher number and altered distribution of PR-positive cells was found (Seagroves et al., 2000). These data suggest that C/EBPβ is involved in the cell fate decision leading to PR expression which then affects cell proliferation through a paracrine mechanism.

Jak2/Stat5 Signaling Controls Cell Specification, Proliferation, and Differentiation

The Jak/Stat signaling pathway exists in most multicellular organisms and has been adapted during evolution to accommodate many diverse cytokine signals (Leonard and O'Shea, 1998; Ihle 2001). Proliferation and differentiation of secretory mammary epithelium is dependent on the presence of the prolactin receptor (PrlR) (Ormandy et al., 1997) and the downstream Jak2/Stat5 pathway (Liu et al., 1997). Binding of prolactin or placental lactogens to the PrlR induces receptor dimerization and the phosphorylation of specific tyrosine residues by receptor-associated Jak2. Subsequently, the transcription factors Stat5a and 5b (the two proteins are 92% identical) are recruited through their SH2 domains and phosphorylated by Jak2. Phosphorylated Stat5a and Stat5b form homo- and hetereodimers and translocate to the nucleus where they activate genetic programs of cell proliferation and differentiation. Deletion of the two *Stat5* genes, individually or together, provided mechanistic insight into this signaling pathway in different cell types, including mammary epithelium and hematopoietic cells. In addition, these experiments suggest that Stat5 function is dosage dependent. Stat5a null mice fail to develop functional mammary tissue during pregnancy as a result of reduced epithelium and impaired differentiation (Liu et al., 1997). After multiple pregnancies, some functional mammary development can be obtained in Stat5a null mice, which coincides with increased Stat5b levels (Liu et al., 1998), suggesting that Stat5b can partially compensate for the absence of Stat5a. Since Stat5a/b null mice have a nonfunctional corpus luteum and are infertile (Teglund et al., 1998), it was not possible to directly assess the combined contribution of both Stat5a and 5b (referred to as Stat5) to the development of mammary tissue during the course of a normal pregnancy. However, this was accomplished through the

transplantation of Stat5 null mammary epithelium into wild-type mice. Stat5 null mammary epithelia developed ducts but failed to form alveoli during pregnancy (Miyoshi et al., 2001), suggesting that the Stat5 pathway is critical for the determination, proliferation, and differentiation of mammary alveoli during pregnancy.

Stat5 controls different developmental processes, but its target genes remain ill defined. A potential target in hematopoietic cells (Socolovsky et al. 1999; Kieslinger et al., 2000) is the *bcl-x* gene, which encodes a cell survival protein. Deletion of the *bcl-x* gene results in impaired erythropoiesis, reminiscent to what has been seen in Stat5 null mice (Socolovsky et al., 1999). However, transcription of the *bcl-x* gene in mammary epithelium is not under Stat5 control (Walton et al., 2001), and unlike Stat5, Bcl-x is not required for normal mammary development and function.

Local Control of Cell Proliferation through the Osteoclast Differentiation Factor RANKL

Mammary development is not only controlled by systemic hormones, but also by peptides produced either in the stromal or epithelial compartment. These include the osteoclast differentiation factor RANKL (Fata et al., 2000), inhibinβB (Robinson and Hennighausen, 1997), and members of the hedgehog (Lewis et al., 1999) and TGFβ families (Nguyen and Pollard, 2000). The TNF family member RANKL (also called osteoprotegerin) was originally identified as a key osteoclast differentiation factor essential for bone remodeling (Kong et al., 1999). RANKL binds to its receptor RANK (receptor activated by NF-κB), which is an intrinsic cell surface determinant that mediates effects on bone resorption. Like PTHrP, RANKL and its receptor RANK control the development of both bone and mammary cells. In the absence of RANKL or its receptor RANK, pregnancy-induced alveolar development is reduced (Fata et al., 2000). Although initial alveolar budding occurs during early pregnancy, expansion and differentiation of the alveoli is arrested. Transplantation of epithelium devoid of the ligand or the receptor into control stroma demonstrated epithelial cell autonomy. Interestingly, while the receptor is expressed throughout development, the ligand is present only after day 12.5 of pregnancy, the time when the developmental defects become apparent. The developmental lesions in RANK and RANKL null mammary epithelium are histologically similar to those observed in both PrlR null and Stat5 null mice, suggesting that the two pathways either operate in parallel or are dependent upon each other. Expression of RANKL can be induced by progesterone, PTHrP, and prolactin, suggesting that it is a downstream mediator of one of these hormones. As activation of Stat5 precedes RANKL expression by approximately 1 day, it is possible that placental lactogens and prolactin are the key inducers. Furthermore, Stat5a activity in mammary epithelium was preserved in RANKL null mice, which excludes RANK signaling as an activator of Stat5.

Several lines of evidence from gene deletion mice suggest that prolactin and RANKL induce identical or related developmental programs during pregnancy. First, the histological and molecular consequences of inactivation of the PrlR and RANK pathway are similar. Second, cell proliferation is impaired in the absence of each pathway. Third, inactivation of both pathways results in increased apoptosis (Humphreys and Hennighausen, 1999; Fata et al., 2000). Additional evidence that the prolactin and RANKL pathways are intimately linked comes from studies of PrlR null mice that exhibit defects in skull bone formation (Clement-Lacroix et al., 1999). The defect is linked to the PrlR in osteoblasts, the same cells that secrete RANKL.

The Cell Cycle

Cyclins activate and provide substrate specificity for cyclin-dependent kinases (Cdks). Three D-type cyclins (D1–D3) are expressed in the G1 phase of the cell cycle, and their expression is largely controlled by extracellular signals, in particular mitogens. Mice in which the cyclinD1 gene has been deleted display several defects, including neurological abnormalities, retinal hyperplasias, and a failure of mammary tissue to fully develop during pregnancy (Sicinski et al., 1995; Fantl et al., 1995). As substantial alveolar proliferation and differentiation remains in the absence of cyclinD1, it is likely that cyclinsD2 and D3 also contribute to the development of the alveolar compartment. In addition to providing insight into the function of cyclins in normal mammary development, these investigations have suggested that cyclinD1 could be a useful target for blocking progression of some mammary tumors. Transgenic mice expressing the neu and ras oncogenes under control of the MMTV-LTR succumbed to mammary tumors within a few months in the presence of cyclinD1 but stayed virtually tumor free in the absence of cyclinD1 (Yu et al., 2001). By contrast, tumors induced by c-*myc* and wnt1 progressed in the absence of cyclinD1. This suggests that cyclinD1 regulates specific growth pathways and that an anti-cyclinD1 therapy might be used to treat certain breast cancers.

Cyclin-dependent kinase inhibitors negatively regulate Cdks and serve as checkpoint controls for cell cycle progression (Sherr and Roberts, 1999). The Cip/Kip family of proteins includes p21, p27, and p57, which inhibit all cyclin-Cdk complexes. p27 binds all cyclinD-Cdk4 complexes and reversibly arrests in the G1 phase of the cell cycle, suggesting that it controls the G1 to S transition. Genetic evidence comes from the deletion of the *p27* gene from the mouse genome, which results in larger mice with hyperplasias in many organs (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996). Based on these observations, it might have been expected that loss of p27 would have resulted in hyperproliferation of mammary epithelium. It was therefore surprising that p27 null mammary epithelium does not develop normally during pregnancy (Muraoka et al., 2001). Instead, during pregnancy p27 null mammary epithelium displays a decreased proliferation rate and delayed differentiation, reminiscent of the situation in cyclinD1 null mice. The combined loss of cyclinD1 and p27 results in overtly normal mammary development (Geng et al., 2001). This restoration of normal mammary development upon deletion of both genes suggests that cyclin D1 and p27 function antagonistically in mammary epithelium. However, since both p27 and cyclinD1 null mammary epithelium both exhibit impaired mammary development, one has to assume that a second, yet to

be defined, pathway is activated in the absence of both proteins.

Transcription factors from the basic helix-loop-helix (HLH) family are critical for cell proliferation and differentiation during developmental processes. Id proteins are negative modulators for these transcription factors. They lack the basic DNA binding domain but retain the HLH dimerization motif and thus function in a dominant-negative fashion. Id2 is essential for the cell population that establishes peripheral lymph organs and mammary development (Mori et al., 2000). Id2 null mammary epithelium exhibits reduced proliferation during early stages of pregnancy, and a lack of functional differentiation and increased rates of apoptosis in late pregnancy. As Id2 first appears in mammary epithelial cells at the time when Stat5 is activated, it is possible that Id2 is a target gene in the prolactin pathway. The impaired cell proliferation in early pregnancy in Id2 null mammary epithelium coincides with increased expression of p21 and p27 (Mori et al., 2000), suggesting that Id2 is also required for cell cycle progression in mammary epithelial cells. Id2 is also a dominant-negative antagonist of the retinoblastoma (Rb) family. Rb null embryos die in utero because of widespread proliferation and defects in neurogenesis and hematopoiesis. Id2-Rb double knockout embryos survive (Lasorella et al., 2000) supporting the concept Id2 is a negative growth regulator. Although Rb is not required for mammary development per se (Robinson et al., 2001), these findings could indicate an integral role for Id2 in the reciprocal regulation of differentiation and proliferation of specific cell types.

Challenges Ahead

Experimental mouse genetics and tissue transplantation approaches have provided mechanistic insight into genes and signaling pathways that are intrinsinc to the mammary epithelium. The best characterized of these are the PrlR-Jak2-Stat5 pathway, the transcription factor C/EBPβ, RANK and its ligand RANKL, and the cell cycle regulators cyclinD1 and Id2. Based on available biochemical and histological tools, it has been suggested that these molecules control the proliferation and differentiation of secretory lobuloalveolar units. However, it is not clear whether these molecules are part of parallel pathways or overlap. Furthermore, it is far from clear which cells types are controlled by the different signals. Ductal elongation and branching occurs during puberty and is accompanied by the transient development of alveolar units during estrus. Although milk proteins can act as markers for differentiating alveolar cells, we need markers that can distinguish undifferentiated and developing alveolar cells from ductal epithelium. At present, if a histological study suggests an underdeveloped gland, it is not clear whether the alveolar lineage failed to proliferate and differentiate or simply was not established. We believe that some of the genes and signaling pathways described in this review do not control proliferation and differentiation of an established cell lineage but instead are intimately involved in cell specification and are required to establish the alveolar lineage. Genetic dissection of the contribution of these genes to proliferation and differentiation will have to come from deletion of genes after the cell lineage has formed. This could be accomplished through the combination of the Cre-loxP-based recombination system tailored to mammary epithelium (Wagner et al., 1997; Xu et al., 1999) with inducible gene switches, such as the tetracycline system (Furth et al., 1994; Ewald et al., 1996; D'Cruz et al., 2001). The physiological and developmental changes observed by conventional gene deletion in mice not only reflect the loss of particular pathways, but also the activity of compensatory pathways and other secondary physiological changes. The true role of any specific protein at any given time point in development can only be established if it becomes possible to delete the respective gene not only in defined cell types but also during predetermined time windows. Only then will we know whether a specific pathway controls cell specification, proliferation, differentiation, maintenance of function, or survival.

Conclusions

The introduction of mammary glands is a recent event in evolution, occurring with the appearance of mammals about 200 million years ago. Many, if not all, of the genetic pathways that control the development of mammary tissue are used in organ systems that appeared earlier in evolution. Thus, the development of mammary glands relied on the recruitment of regulators from other sources, such as epidermal appendages, bone morphogenesis, reproduction, and hematopoiesis, and their subversion into signaling components for a new and unrelated cell type. It is intriguing to speculate that the salvage and recycling of signaling switches from cell systems that are constantly renewed or remodeled might have facilitated the establishment of this new organ.

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